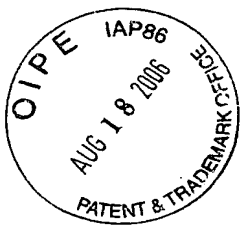
APPENDIX C



Detection of Head and Neck Squamous Cell Carcinoma among Exfoliated Oral Mucosal Cells by Microsatellite Analysis[1]

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ABSTRACT

Prompt detection of head and neck squamous cell carcinoma (HNSCC) is vital to successful patient management. In this feasibility study, we used microsatellite analysis to detect tumor-specific genetic alterations in exfoliated oral mucosal cell samples from patients with known cancer. Exfoliated mucosal cells in pretreatment oral rinse and swab samples were collected from 44 HNSCC patients and from 43 healthy control subjects (20 nonsmokers and 23 smokers). We tested a panel of 23 informative microsatellite markers to assay DNA from the matched lymphocyte, tumor (from cancer cases), and oral t est samples. Loss of heterozygosity or microsatellite instability of at least one marker was detected in 38 (86%) of 44 primary tumors. Identical alterations were found in the saliva samples in 35 of these 38 cases (92% of those with markers; 79% overall) including 12 of 13 cases with small primaries [stage T_1 or T_x (occult primary)] and 4 of 4 cases of patients that had undergone prior radiation. Microsatellite instability was detectable in the saliva in 24 (96%) of 25 cases in which it was present in the tumor, and loss of heterozygosity was identified in the test sample in 19 (61%) of 31 cases. No microsatellite alterations were detected in any of the samples from the healthy control subjects. This approach must now be refined and validated for the detection of clinically occult disease. Microsatellite analysis of oral samples may then become a valuable method for detecting and monitoring HNSCC.

INTRODUCTION

HNSCC[3] affects 50,000 Americans and >500,000 individuals worldwide each year (1). Early detection can improve patient survival and diminish the morbidity of treatment required for advanced disease. However, early detection is hindered by several factors: (*a*) risk factors (tobacco and alcohol use) and early warning signs (such as hoarseness and otalgia) are not universally understood; (*b*) patients often delay seeking medical attention for a variety of psychological and social reasons; (*c*) examination of the upper aerodigestive tract requires expertise and equipment not possessed by many clinicians; and (*d*) even when a provider has the necessary capability, a tumor may remain undetected because many head and neck cancers occur in hidden sites such as crypts in the base of tongue or tonsil, or beyond view in the hypopharynx and larynx. Failure to diagnose HNSCC in its earliest stages is perhaps the greatest factor contributing to the poor outcome for treatment of this disease. In the larynx, for example, stage I squamous cell carcinoma is successfully treated in 90% of cases. In contrast, less than 50% of stage IV tumors are controlled, often with loss of the larynx (2). Prompt detection is also vital to effective surveillance after treatment of HNSCC. The opportunity for successful surgical salvage after chemotherapy and/or radiation therapy is lost if the residual cancer grows or spreads while remaining hidden amid scar or inflammation. Beyond clinical examination, no early detection technique currently exists for squamous cell carcinoma of the upper aerodigestive tract. Innovations in early detection technology offer a means to reduce morbidity and mortality of HNSCC using current treatment options.

Microsatellite alterations have been used as markers of clonality (3) and to detect cancer cell DNA in a background of normal cells (4). Microsatellite analysis can reveal either LOH or MSI in the amplified microsatellite repeat locus. Tumor-specific DNA alterations can be found in the body cavity fluids and blood of patients with various cancers. For example, tumor-specific microsatellite alterations can be consistently detected in the urine of patients with bladder carcinomas (5) and can be detected in the serum of a significant percentage of patients with HNSCC (6). The purpose of his study was to determine whether tumor-specific microsatellite alterations may also be detectable in the DNA from exfoliated oral cells in saliva of HNSCC patients. We planned to assess a panel of microsatellite markers in a group of subjects with clinically apparent cancer and healthy control subjects as a first step in the development of a screening tool.

Received 9/15/00; revised 11/27/00; accepted 11/28/00.
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[1] Supported by NIH/National Institute of Dental and Craniofacial Research Grants RO1DE012588-01 and RO1 DE13152-01.
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[3] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; LOH, loss of heterozygosity; MSI, microsatellite instability.

Table 1 Tumor characteristics

Tumors from 44 patients with HNSCC were analyzed at 23 microsatellite loci and results were compared with DNA from peripheral blood lymphocytes. Exfoliated oral mucosal cells were also analyzed with the same panel of microsatellite markers. The location of the tumors was primarily in the oral cavity and oropharynx, accessible to the rinse/swab harvesting of exfoliated cells. The T stage reflects the surface area of the tumors in general and is distributed from clinically inapparent (T_x) to extensive T_4 lesions.

	n	Marker in tumor	Marker in oral sample
Site			
Oral cavity	13	11	11
Oropharynx	22	18	15
Larynx	5	5	5
Hypopharynx	1	1	1
Unknown	3	3	3
T stage			
T_1	13	10	9
T_2	8	7	6
T_3	5	5	4
T_4	11	10	10
rT_4[a]	4	3	3
T_x	3	3	3

[a] rT_4, recurrent T_4.

MATERIALS AND METHODS

Patients

The study involved 44 patients with HNSCC and 43 healthy control subjects including 20 nonsmokers and 23 smokers. All of the smoking control subjects had at least a 20-pack-year smoking history and continued to smoke at the time of sample collection. The majority of cancer subjects had newly diagnosed HNSCC, although six patients were entered into the study at the time of diagnosis of a recurrent cancer or second primary cancer after previous radiation therapy. Each of the individuals in the control group underwent a physical examination by a head and neck surgeon, including inspection of the oral cavity, pharynx, and larynx to ensure that no suspicious mucosal lesion was present. An Institutional Review Board-approved informed consent was obtained from each participant. Table 1 provides the site and stage of the tumors included in the study.

Tissue Collection

All of the samples were collected by a head and neck surgeon (M. F. S., W. M. K.). Tumor and blood were obtained from each HNSCC patient at the time of biopsy or resection. In three cases, the tumor sample was taken from a metastatic lymph node of a patient who had presented with cancer arising in an unknown mucosal primary site (unknown primary). Fresh tumor tissue obtained at surgery was used when available. In six cases, the tumor was microdissected from paraffin-embedded tissue. Ten ml of blood were collected in citrated tubes as a source of normal control DNA. Patients contributed a pretreatment oral rinse by swishing and gargling for 15 s with 25 ml of sterile 0.9% NaCl. Then the oral cavity was swabbed by the surgeon using a cotton-tipped applicator. Three strokes were performed of each buccal surface, the alveolar ridges, lateral tongue, floor of mouth, and pharyngeal inlet (tonsil, soft palate, and posterior tongue). The applicator was rinsed in saline after stroking each region. At the beginning of the study, the rinse and swab material was combined immediately, and analyzed together. Later, the rinse and swab samples were kept separate, and analyzed individually. All of the results will be reported as combined samples because there was no significant advantage seen in the analysis of separate rinse and swab specimens. In six cases, the tumor involved a site (five larynx, one hypopharynx) that would not have been sampled under the protocol of the swabbing just described. The test sample in these cases was taken at the time of examination under anesthesia, and included a swab of the hypopharynx and supraglottic larynx. Blood and oral rinse-swab samples were also collected from the 43 control subjects. All of the oral samples were collected in a sterile, closed container, refrigerated immediately, and processed to collect DNA within 8 h.

DNA extraction

Tumor

Frozen Tissue. Representative thin tumor sections were stained with H&E. Microdissection was performed as needed, removing noncancerous tissue to achieve at least a 70% purity of the neoplastic cell population. Ten 12-μm tissue sections were placed in 1% SDS/proteinase K (0.5 mg/ml) and incubated at 48°C for 72 h.

Paraffin-embedded Tissue. Representive tumor samples were sectioned producing 25 14-μm samples. They were placed on glass slides and microdissected using a dissecting microscope. The samples were placed in xylene overnight for deparaffinization, pelleted in 70% ethanol, dried, and incubated in SDS/proteinase K at 48°C for 72 h.

Blood

Ten ml of blood were brought to 30 ml with TM buffer (2% Tris-EDTA-MgCl). Lymphocytes were collected by centrifugation at 2500 rpm for 15 min and were placed in 1% SDS/proteinase K (0.5 mg/ml) at 48°C for 72 h.

Exfoliative Oral Cells

The 25-ml rinse and swab (test) samples were subjected to centifugation at 2500 rpm for 15 min. The supernatant was discarded, and the cell pellet was retained and placed in 1% SDS/proteinase K (0.5 mg/ml) at 48°C for 72 h. Digested tissue and fluids from all of the sources were then subjected to phenol-chloroform extraction and ethanol precipitation.

Microsatellite Analysis of DNA

A panel of twenty-three tetranucleotide microsatellite repeat PCR primers (Research Genetics, Huntsville, AL) that had been used in similar studies in our laboratory were selected for this study (Table 2). Several of these alterations were known to be highly informative because of a study of the allelotype of HNSCC (6). Others were identified in studies of lung (7) and bladder carcinoma (8). This experience indicated a high frequency of MSI in microsatellites consisting of $(AAAG)_n$ repeats, and, therefore, the panel preferentially included those markers. Prior to amplification, 50 ng of one primer from each pair was end-labeled with [γ-^{32}P]ATP (20 mCi; Amersham Life

Table 2 Microsatellite panel

A panel of 23 microsatellite sequences were used in an effort to identify at least one tumor-specific marker for each of 44 HNSCC lesions. The panel includes markers from chromosomal loci known to have a high likelihood of allelic loss in HNSCC, and many $(AAAG)_n$ sequences. These tetramers were particularly useful with a high rate of MSI in the tumors.

Microsatellite	Location	Repeat sequence	No. of markers, T/S[b]	
			LOH	MSI
D9S753	9q21.1–22.3	$(A)_5(AAAG)_{18}(AGAG)_4(A)_9$	1/1	6/4
D20S77	20	$(AAAG)_{21}$	0	2/1
UT5307	8	$(AAAG)_{19}$	1/0	1/1
D9S242	9q32–33	$(AAAG)_{26}(AAAG)_3$	1/0	1/1
CSFIR-6	5q33.4–34	(TAGA)	2/1	1/1
D11S488	11q24.1–25	$(AAAG)_{14}(GAAG)_{10}$	4/1	1/1
ACTBP2	5	$(AAAG)_{26}$	5/2	1/1
D8S321	8q24.13–8qter	$(AAAG)_{12}$	1/0	3/1
UT5320	8	$(AAAG)_{10}$	2/1	3/2
D9S171	9p21	$(A)_{10}$	2/2	3/2
D9S162	9p22–21	$(CA)_{24}$	2/2	2/1
D20S82	20	$(AAAG)_{10}$	1/0	1/0
D20S85	20	$(AAAG)_{12}$	1/1	0
Li7686	7q31–32	$(AAAG)_{30}(AAGG)_{22}$	0	5/4
FGA	4q28	$(TCTT)_{13}$	2/1	4/3
D9SIFNA	9p22	$(CA)_n$	7/0	2/2
D11S654	11p12–11p11.2	Unknown	2/0	1/1
D3S1560	3p26–3p25	$(AC)_{22}$	2/1	2/1
D3S1286	3pter–3p24.2	$(T)_5$	7/4	3/3
D3S1289	3p23–3p21	$(CA)_{23}$	5/1	5/4
D17S695	17	$(AAAG)_n$	3/3	1/1
D17S654	17	$(CA)_n$	5/3	1/1
D17S656	17	Genbank accession no. 2574	1/0	1/0

[a] Locations listed as chromosome number only have no more specific information available in current web genomic data bases.
[b] T, tumor; S, saliva.

Sciences, Inc., Arlington Heights, IL) and T4 kinase (New England Biolabs, Inc., Beverly, MA) in a total volume of 50 μl. PCR reactions were carried out in a total volume of 12.5 μl containing 10 ng genomic DNA, 0.2 ng labeled primer, and 15 ng of each unlabeled primer. The PCR buffer included 16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM magnesium chloride, 10 mM β-mercaptoethanol, and 1% DMSO, to which were added 1.5 mM deoxynucleotide triphosphates and 1.0 unit Taq DNA polymerase. (Boehringer-Mannheim Biochemicals, Indianapolis, IN). PCR amplifications of each primer set were performed for 30–35 cycles consisting of denaturation at 95°C for 30 s, annealing at 50–60°C for 60 s, and extension at 72°C for 60 s. One-third of the PCR products were separated on 8% urea-formamide-polyacrylamide gels and exposed to film from 4 to 48 h. For informative cases, allelic loss was documented if one allele was significantly decreased (>50%) in tumor- or oral-test DNA compared with the same allele in the normal (lymphocyte) DNA. MSI was described if an additional band representing a change in repeat number was noted in tumor- or oral-test DNA. All of the samples were assessed by two observers independently (M. F. S., D. S.) and borderline cases were decided by densitometry. All of the identified alterations were confirmed by repeating the PCR reaction and electrophoresis. Samples were coded, so that the observers were blinded to the individual subject.

RESULTS

Thirty-eight of 44 tumors (86%) displayed a microsatellite alteration in at least one locus (Table 3). Twenty-five displayed MSI and 31 had LOH at an examined locus. Eighteen tumors displayed both MSI and LOH, 13 had LOH only and 7 had MSI only. Thirty-one tumors had microsatellite alterations at more than one locus, but 7 tumors displayed changes in only one marker. All of the 23 microsatellites were altered in at least one tumor. With this panel, the average number of alterations per tumor was 2.7 (range, 0–13) with an average of 1.5 instances of LOH (range, 0–5) and 1.2 of MSI (range, 0–13). Thirteen markers displayed MSI in two or more tumors (maximum, six tumors). Five microsatellites were the only markers altered in at least one tumor (Table 2).

Table 3 Number of tumors with LOH and MSI

Two by two tables demonstrate the number of tumors and oral samples with genetic alterations at various microsatellite loci. Tumors (6) lacking both MSI and LOH had no markers available for screening the oral exfoliative samples.

	Tumors with MSI		
	No	Yes	
Tumors with LOH			
Negative	6	7	13
Positive	13	18	31
	19	25	44

None of the oral specimens of the tumor-free controls displayed a microsatellite alteration. In contrast, in 35 (92%) of 38 cancer cases for which a tumor marker was available, at least one genetic alteration matching those seen in the tumor was also

Table 4 Number of tumors and oral samples with any microsatellite alteration

Of 38 cases in which the tumor had at least one microsatellite marker, cells with the same alteration were identified in the test sample in 35.

	Tumors with alteration		
	No	Yes	
Oral sample			
Negative	6	3	9
Positive	0	35	35
	6	38	44

Table 5 Number of tumors and oral samples with LOH

At least one marker with LOH was present in the tumor in 31 cases and could be identified in a test sample in 19 cases.

	Tumors with LOH		
	No	Yes	
Oral sample			
Negative	13	12	25
Positive	0	19	19
	13	31	44

seen in an exfoliative oral-test specimen (35/44, 79% overall; Table 4). In cases for which the oral rinse and swab specimens were analyzed separately, tumor DNA was detected in 57% of the swab samples compared with 44% of the rinse samples. This difference in rate of detection was not statistically significant. All of the three cases displaying tumor markers that were not detected in the oral exfoliative sample harbored tumor in the oropharynx, one involving the tonsil, one, the base of tongue, and one, the posterior pharyngeal wall (staged T_3, T_1, and T_2, respectively). In two of these cases, only one marker with LOH was found in the tumor, and, therefore, only one was potentially available for detection.

MSI is more easily recognized amid a background of normal cell DNA than is LOH. As a result, MSI was more efficient for tumor cell detection in the oral samples. Of the 31 cases in which a tumor displayed LOH, the same loss was detected in the matching test sample in 19 cases (61%; Table 5). In contrast, MSI was detected in the test specimen in 24 (96%) of 25 cases in which a shifted marker was present in the tumor (Table 6). Overall, MSI contributed 64% of the positive tumor-test matches in the sample set.

Twelve (92%) of 13 small primary (T_x and T_1) lesions for which a microsatellite marker was identified in the tumor were also detected in the test sample (12 of 16 overall; Table 1). These 12 included the three cases that presented with metastatic HNSCC in cervical lymph nodes without a detectable primary lesion. In all three of the cases, cells matching the metastatic tumor were detected in the test sample. Nine of 10 T_1 tumors were also detected, including one located in the supraglottic larynx. The relatively small size of T_x and T_1 primary lesions did not adversely influence the likelihood of detecting a LOH marker in saliva. Of the 13 T_x and T_1 lesions displaying at least one LOH marker, the same alteration was detected in saliva of 6 (60%).

Table 6 Number of tumors and oral samples with MSI

At least one shift marker was present in the tumor in 25 cases and could be identified in the test sample in 24 cases.

	Tumors with MSI		
	No	Yes	
Oral sample			
Negative	19	1	20
Positive	0	24	24
	19	25	44

Index microsatellite alterations were identified in the tumor of 4 of 6 patients entered in the study after prior radiation therapy. Matching alterations were detected in exfoliative oral samples in all four of the cases.

DISCUSSION

Early cancer detection in body fluids such as urine or sputum typically requires morphological identification of a few neoplastic cells in a background of normal epithelial cells. Standard cytological methods have been shown to have potential value for the detection of second primary tumors in the esophagus of patients with HNSCC (9), and have been assessed for the early detection of lung cancer (10). We did not subject our samples to cytological evaluation, although the dental literature of the 1960s and 1970s contained numerous reports on the use of oral cytology as a diagnostic approach. However, low sensitivity and specificity precluded the general adoption of microscopic cytology for the detection of primary or recurrent HNSCC (11, 12, 13). Cytological evaluation is labor intensive, requiring a high degree of experience to accurately identify morphologically suspicious cells. In contrast, assessment of molecular alterations is less subjective, relying on the identification of tumor-specific changes in the DNA. This approach is also amenable to automation using recent innovations in microcapillary array technology (14). In our hands, molecular markers displaying instability have proven to be highly sensitive, able to detect a single tumor cell out of 200 normal cells in the urine; however, LOH markers are much less sensitive, able to detect only one neoplastic cell among 3–4 normal cells (5). In patients with bladder cancer, large amounts of neoplastic cells are shed into the urine. We were concerned that the rate of normal cell turnover in the oral cavity would produce more exfoliation of normal cells obscuring the detection of cancer beyond that seen in the bladder. However, the results of this study have shown that microsatellite analysis (both LOH and MSI) can be used to detect DNA from exfoliated tumor cells in the saliva of cancer patients.

The panel of 23 microsatellites identified at least 1 altered marker for all but 6 of the tumors in this series. Only tumors that displayed alterations at one or more of the loci could be detected in an exfoliative sample, which excluded 14% of tumors from the analysis. When a tumor marker was available, exfoliated cancer cells were detectable in the oral specimens from the vast majority (92%) of cases. Failure to detect tumor cells in three

cases displaying a tumor-specific marker may be attributed to a number of factors, such as necrotic tumor yielding little DNA, a high background of normal epithelial cells, mostly submucosal tumor growth, problems with sample handling and processing, or the type (LOH *versus* MSI), and quantity of altered markers.

Previous studies have demonstrated the capability of scrapings from oral lesions (15) and cytological brushings of laryngeal neoplasms (16) to produce cells with tumor-specific microsatellite alterations. Our protocol included swabbing the tumor bed, but we did not vigorously scrape or brush the index lesion when it was visible. Instead, the harvesting of exfoliative cells was performed in a manner intended to simulate a potential screening protocol. Our population included six cases in which the tumor was not in a region that could be sampled in an office-based swab protocol. The hypopharynx and larynx are easily sampled under general anesthesia, and exfoliative samples from these areas might be obtained in a manner similar to "induced sputum" cytology (17). If these cases were eliminated from the analysis, the protocol was able to detect cancer in 29 (90%) of 32 oral and oropharyngeal cases for which a tumor marker was available, or 76% of the remaining population of 38 patients overall.

This work constitutes a feasibility trial aiming to demonstrate proof of principle for a molecular detection approach using our panel of microsatellite markers in patients with obvious HNSCC. If the test is validated and refined, molecular analysis of exfoliated oral mucosal cells may be useful as a detection method for patients at risk for developing HNSCC *de novo*, and as a surveillance tool for patients after completion of therapy. A swab of the tumor bed could be performed after treatment for tumor surveillance, but swabbing the tumor will not be possible when the saliva test is applied to screen at-risk individuals without clinical lesions. An indication of the applicability of the saliva test for screening the at-risk population is found in the successful test results of the three patients with metastatic HNSCC from unknown mucosal primary sites. Oral rinses from these patients produced exfoliated cancer-related cells in each case. Although the oral sample could not identify the site from which the clonal cells originated, it could identify their presence. Directed biopsies of commonly involved mucosal sites are typically harvested in the work-up of patients with HNSCC of unknown primary. These could then be analyzed both histologically and using microsatellite analysis. We have reported the utility of molecular testing to identify cancer cells in histologically benign directed biopsy samples from such patients (18).

Radiation therapy might prohibit the application of the test in the surveillance setting by altering the rate of exfoliation of both normal and cancer cells in the upper aerodigestive tract or by interfering with the identification of microsatellite alterations. However, successful detection of tumor cells was possible in the test samples of all four of the patients who had undergone previous radiation therapy and who had a marker identified in their tumor. These results support the potential application of the test in postradiation therapy surveillance.

Our panel of markers has been developed with an emphasis on MSI detection and known areas of LOH, featuring a majority of tetranucleotide repeat markers with the $(AAAG)_n$ repeat motif (Table 2). It was not our goal to streamline the panel of markers at this stage of test development. Given the limited sample size of this pilot cohort, a marker altered in even one tumor was considered potentially valuable. Five microsatellites were the sole markers available for at least one tumor and only two microsatellite alterations had no positive matches between tumor and test samples. Thus, the panel contained few, if any, unnecessary markers. For example, one marker (D20S85), that was detected in only one tumor was also the only available marker for that tumor and was detected in the test sample as well. Markers that display MSI were found to be better than LOH markers for detecting tumor cells in a background of normal cells. Relatively pure clonal populations of tumor cells are required for detection of LOH, because the loss of the signal may be obscured easily by the presence of the alleles in the normal cell majority. Microsatellite loci that display MSI are valuable because the amplified novel tumor DNA band separates from maternal and paternal alleles on gel electrophoresis and serves as a unique positive-signal clonal marker for the tumor and its exfoliated daughter cells. Thirteen markers displayed MSI in multiple tumors. These microsatellites are arguably the most valuable in the panel. If altered in tumor, they were detected in saliva 66% of the time and account for 47% of all of the positive saliva results in the entire study.

Future refinement of the approach may focus on several areas. More microsatellite markers could be added to the panel, thus increasing the yield of detectable tumors above the current 86%. In particular, tetranucleotide markers displaying MSI in a high proportion of tumors seem to be promising candidates for addition to the panel. Tumor-specific promoter methylation markers may also be useful (19). Microcapillary array technology now allows more rapid and efficient screening of large numbers of samples (12).

The molecular assessment of exfoliated cells from oral rinsing/swabbing must now be tested in a prospective, blinded fashion in clinical settings requiring actual cancer detection. The fact that no healthy control subject had any DNA alteration is encouraging, indicating the excellent specificity of the microsatellite analysis. Normal appearing mucosa in patients with early cancer or premalignant lesions has been shown to harbor occult microsatellite alterations (20). It is precisely in these patients that the value of detecting asymptomatic cancers by microsatellite analysis must be prospectively assessed, a setting requiring a highly specific test. A saliva test could be administered by nonspecialists in remote locations as a screening tool to select patients for referral for careful evaluation of the upper aerodigestive tract. Finding early stage, previously undetected disease and prompt identification of persistent disease after therapy using sensitive microsatellite analysis may ultimately save lives.

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